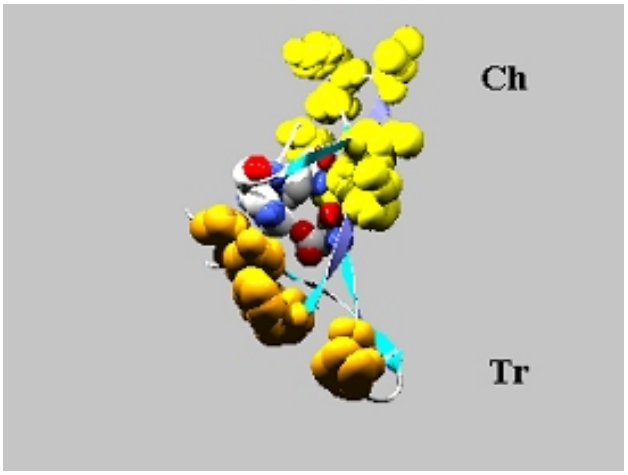

The exposed hydrophobic patch belonging to the trypsin-inhibitory region (Tr) is shown in ochre and that projecting out from the chymotrypsin-inhibitory region (Ch) is shown in yellow. Residues belonging to the buried polar interior are highlighted in CPK.

tion. The appearance of apparently metastable state supports a kinetic reaction control for the variants on the template although it cannot rule out a thermodynamic reaction control. In fact, the template facilitates folding not only kinetically, by reducing the high barrier of activation in solution but also thermodynamically by stabilising the fully active state by means of protein-protein-interactions. Protein metastability has also been documented for certain proteolytic enzymes after removal of their prosequences and for the native conformation of viral hemagglutinins. Presently, it is unknown whether the native conformation of soybean BBI corresponds to a global energy minimum or a metastable state on its conformational landscape. However, the inside-out situation in BBI and the conformational changes that are induced with reducing agents even in the absence of denaturants seem to be more in favour of the second possibility.

for full paper see page

SATURATION MUTAGENESIS OF L177 IN HALOALKANE DEHALOGENASE LINB

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Enzyme LinB is the haloalkane dehalogenase from bacterium *Sphingomonas paucimobilis* UT26. It is involved in a biochemical pathway for degradation -hexachlorocyclohexane. LinB catalyses hydrolytic dehalogenation of broad range of halogenated aliphatic compounds [1]. The amino acid in position 177 was identified as a very important determinant of catalytic properties of LinB by structural analysis [2] and by comparison of its protein sequence with other family members: (i) L177 is positioned in the mouth of the entrance tunnel leading the enzyme active site and is pointing directly to the tunnel and (ii) L177 is the most variable residue of the active site pocket among different haloalkane dehalogenases. L177 of the wild type enzyme was therefore replaced by every other amino acid and then the effect of mutations on enzyme activity was studied. Construction of the protein variants was conducted in two successive rounds. In the first round, L177 was replaced by A, C, G, F, K, T and W, respectively. The specific activities of the first set of mutants were statistically analysed but results from this analysis were not statistically significant. For that reason the first set of mutants was complemented with the second set of mutants comprising replacement of L177 by D, E, H, I, M, N, P, Q, R, S, V and Y, respectively.

All seven protein variants of the first set could be overexpressed in *Escherichia coli* and showed activity with at least some of the substrates used for characterization. In the second set, two out of twelve protein variants (L177E and L177N) could not be overexpressed in *E. coli*, while other two variants (L177P and L177I) did not show activity with any of the substrates. Circular dichroism spectra were recorded for all proteins purified in the second set and two inactive mutants showed spectra different from wild-type LinB and other mutants (Fig. 1), suggesting a decrease in the number of amino acids in -helical conformation and protein unfolding.

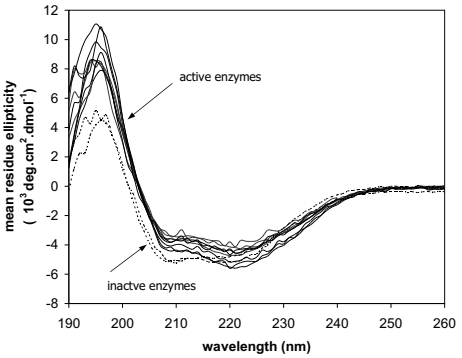

Fig. 1. Far-UV circular dichroism spectra of the wild-type haloalkane dehalogenase LinB, L177D, L177H, L177M, L177Q, L177R, L177S, L177V, L177Y mutants (solid lines) and L177P, L177I mutants (dashes lines). The spectra were measured at room temperature with the protein concentration 0.3 mg/ml in 50 mM phosphate buffer (pH 7.5) using the JASCO J-810 spectropolarimeter.

Successfully purified enzymes from both sets were kinetically characterized using a gas chromatography. Compounds 1-chlorobutane and 1,2-dibromoethane were selected as the substrates for steady-state kinetic measurements because they often serve as the reference compounds for characterization of the haloalkane dehalogenases. Dehalogenation of 1-chlorobutane showed typical Michaelis-Menten dependence, while dehalogenation of 1,2-dibromoethane showed substrate inhibition. Furthermore,

the specific activities of all prepared enzymes were determined for twelve different substrates (1-chlorobutane, 1-chlorohexane, 1-bromobutane, 1-iodobutane, 1,2-dichloroethane, 1,2-dibromoethane, 1,3-diidopropane, 1,2-dichloropropane, 1,2,3-trichloropropane, chlorocyclohexane, bromocyclohexane and 3-chloro-2- methyl propene) and statistically analysed by Principal Component Analysis. The first and only important component explained 45.8% of the data variance. Catalytic activity of mutant protein correlated mainly with the size of amino acid introduced to the position 177.

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STUDY OF ENZYME CATALYSIS USING TRANSIENT KINETIC AND MICROCALORIMETRY TECHNIQUES

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Kinetic studies in enzymology deals with elucidation of enzymatic reaction pathway, identification of reaction intermediates and with specification of the steps that limit the rate of turnover. The kinetic analysis of an enzyme mechanism often begins by analysis of the steady-state kinetics. The steady-state kinetic parameters, K_m and k_{cat}, provide information sufficient to describe a minimal kinetic scheme. Conclusions that can be derived by steady-state analysis are considered preliminary. Because the steady- state kinetic parameters are complex functions of all the reactions occurring during enzymatic catalysis, individual reaction steps are buried within these terms and cannot be resolved. These limitations are overcome by examination of the reaction pathway by transient kinetic methods, where the enzyme is examined as a stoichiometric reactant, allowing individual steps in pathway to be established by direct measurement. Steady-state and transient-state kinetic studies complement each other in elucidation of enzymatic reaction pathway. Analysis in the steady-state should be a prelude to the proper design and interpretation for more detailed transient-state experiments [1].

Both steady-state and transient-state kinetic methods were applied to solve reaction pathway, to identify reaction intermediate and to specify the rate limiting step of catalytic action of haloalkane dehalogenase LinB from bacterial strain *Sphingomonas paucimobilis* UT26 [2]. Steady-state experiments involved direct monitoring of LinB activity by isothermal titration calorimetry and initial rate of product formation measurements by gas chromatography. Transient-state kinetics used stopped-flow fluorescence and rapid-quench-flow techniques. Additional steady-state inhibition experiments and transient-state binding experiments were employed to find out leaving ability of both products (a halide and a alcohol) during dehalogenation reaction.

The results showed that export of products as well as import of substrates into the active site of LinB are fast processes reaching rapid equilibrium. This fast exchange of the ligands between the active site and bulk solvent can be explained by wide opening of the entrance tunnel and large active site of LinB. In contrary, the release of the halide ion from narrow active site after the reaction was found to be slow rate limiting step for another haloalkane dehalogenase, enzyme DhlA from *Xanthobacter autotrophicus* GJ10 [3]. The actual cleavage of the carbon-halogen bond was found to be fast step in both enzymes.

The rate of cleavage of C-Br bound is faster than cleavage of C-Cl bound, which is in agreement with bromide being a better leaving group in biomolecular nucleophilic substitution than chloride. This observation correlates with the lower affinity of LinB for chloride compared to bromide. Further the results confirmed, that the reaction proceeds via a covalent alkyl-enzyme intermediate. Using bromocyclohexane, chlorocyclohexane and 1-chlorohexane as model substrates, hydrolysis of this intermediate was found to be the slowest step in the catalytic cycle of LinB. The alkyl-enzyme complex was highly accumulated due to the fast dehalogenation step following the slow hydrolyses of this intermediate. The study provides a basis for the analysis of kinetic steps in hydrolysis of environmentally important substrates by the action of LinB.

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